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Disaccharide supplementation of extenders is an effective means of improving the cryopreservation of semen in sturgeon

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ABSTRACT

Sperm damage can be alleviated by optimizing the types and levels of cryoprotectants (including sugars) added to the extender to protect the sperm cells during freezing. This study was conducted to elucidate the effect of different disaccharides (maltose, lactose, trehalose and lactulose) upon the motility parameters of cryopreserved semen and the post-thaw storage of semen from Persian sturgeon, *Acipenser persicus* and Beluga sturgeon, *Huso huso*. Semen samples were diluted 1:5 in 9% methanol containing 0.2 M maltose, trehalose, lactose or lactulose. Then, semen was equilibrated for 10 min on ice and submerged in liquid nitrogen. After thawing (40 °C, 5 s) semen was stored for 0, 15, 30 and 60 min at 4 °C and semen motility investigated. For both sturgeon, the type of disaccharide had no effect on the duration of semen motility (expressed as curvilinear velocity, VCL). From the four disaccharides tested, lactulose exhibited the lowest sperm percentage. Furthermore, for all four disaccharides, cryopreserved semen motility was not affected by storage times of up to 30 min maximum; however, after 60 min of storage, a significant reduction was observed. Consequently, the best results were achieved with the disaccharides featuring glucose in their composition. In other words, maltose, lactose and trehalose can be used successfully for the cryopreservation of sturgeon semen.

1. Introduction

The protection of endangered species, such as Persian sturgeon (*Acipenser persicus*) and Beluga sturgeon (*Huso huso*), is often hindered by a lack of male gametes during the breeding season and an inability to preserve germplasm resources over the long-term. Cryopreservation is a well-known means of preserving germ-plasm and it has applications in animal husbandry, aquaculture, biotechnology and the conservation of threatened species (Holt, 1997). Cryopreservation of semen can guarantee a constant supply for the conservation of genetic diversity and the genetic improvement of animal resources (Cabrita et al., 2010), and it also provides reliable technologies for the conservation of certain endangered sturgeon species and for the artificial propagation in sturgeon hatcheries (Billard et al., 2004). However, cryopreservation exposes the sperm membrane to cold shock and oxidative attack, which reduce semen survival and fertilizing ability, thus leading to semen death and a negative impact on the preservation of semen for artificial insemination (Evans, 1988; Maxwell and Watson, 1996). Thus, there are many weaknesses in the semen cryopreservation process that can be improved in order to provide good quality frozen–thawed fish semen. In order to overcome these obstacles, different additives have been added to the extenders used to dilute semen during cool-storage. The basic ingredients of the extenders used to freeze semen now are the same as those used years ago, including the cryoprotective agents glycerol, egg yolk and sugars (Cabrita et al., 2010). Thus, the choice of cryoprotectant is a vital factor underlying the success of fish sperm cryopreservation.

Cryoprotectants can be divided into two groups: permeable and non-permeable. Those in the first group are capable of permeating the cell membrane; these agents are generally of low molecular weight (dimethyl sulfoxide, methanol or glycol). The second group of cryoprotectants consists of non-permeable agents of high molecular weight (sugars, egg yolk or vegetable oils) (Moraes et al., 1998; Tiersch, 2011). Sugars can be categorized into three groups as either monosaccharides (glucose, fructose or galactose), disaccharides (maltose, lactose or trehalose) and polysaccharides (starch). Sugars are known to stabilize membranes by interacting with the polar head groups of plasma membrane phospholipids at low hydration to form a glass (vitrification), by depressing the membrane phase transition temperature of dry lipids, and by providing energy for semen during incubation (Aisen

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et al., 2002). Sugars can also maintain the osmotic pressure of diluents, induce cell dehydration, and reduce the incidence of ice crystal formation in the semen (Purdy, 2006). Some studies have shown that cryodamage resulting from freezing and thawing semen can be minimized by adding these non-permeable cryoprotectants (reviewed by Cabrita et al., 2010).

The efficacy of glucose (monosaccharide) and sucrose (disaccharide) as cryoprotective supplements have shown particular promise, as these typically increase cell survival after freeze thawing. These sugars have also been widely applied in the cryopreservation of sturgeon semen (Tsvetkova et al., 1996, Billard et al., 2004, Boryshpolets et al., 2011; Judycka et al., 2015; Aramli et al., 2015). In the case of other disaccharides, trehalose is found in a number of plants and animals that can resist dehydration or freezing; it can form hydrogen bonds with the polar head groups of phospholipids and therefore helps to prevent fusion events in juxtaposed membranes (Woelders et al., 1997). Trehalose has been reported to improve post-thaw semen quality in mammals (Aisen et al., 2002; Hino et al., 2007; Gutierrez-Perez et al., 2009; Oh et al., 2012), and certain types of fish (Miyaki et al., 2005; Lichtenstein et al., 2010; Nynca et al., 2016), but it has not been studied in sturgeon, yet. Maltose, lactose or lactulose were also highly effective in protecting mammalian semen during cryopreservation (Gomez-Fernandez et al., 2012), although there are no reports of its use in fish and sturgeon. In addition, it is unknown whether the substitution of glucose with different disaccharides (except for sucrose) can improve the cryopreservation procedure and lead to higher post-thaw sturgeon sperm motility just after thawing or during post-thaw storage.

Therefore, the first objective of the present study was to test the efficacy of different disaccharides (maltose, lactose, trehalose and lactulose) on sperm motility characteristics and the fertilization ability of cryopreserved semen from Persian sturgeon and Beluga sturgeon. The second objective was to test the effect of extenders on the duration of post-thaw semen storage in these species.

2. Materials and methods

2.1. Regents

All chemicals in the present study were purchased from Sigma-Aldrich Chemical Company (Sigma, St. Louis, MO, USA) unless stated otherwise and prepared with purified water (18 MΩ cm; Automatic GR Wasserlab, Spain).

2.2. Broodfish condition

The male and female Persian sturgeon (mean weight, 30–40 kg; total length, 1–2 m) and Beluga sturgeon (mean weight, 35–45 kg; total length, 1.5–2 m) used in this study were obtained from the Rajaeei Sturgeon Hatchery Center (Sari, Iran.(The mean water quality parameters were as follows: temperature 15–16 °C, dissolved oxygen > 5 mg/l and pH 7.6–7.9. All fish manipulations were conducted in accordance with the guidelines on the care and use of animals for scientific purposes (National Health and Medical Research Council, Australia).

2.3. Gamete sampling

Spermiation was stimulated by a single intramuscular injection of a luteinizing hormone-releasing hormone agonist at 5 μg/kg body weight (Nazari et al., 2010) 16 h before semen collection. Semen was collected from the urogenital papilla by aspiration through a plastic catheter (5–7 mm diameter) connected to a 50 mL syringe. Special care was taken to avoid contamination with mucus, feces, or water.

Females were injected with the same hormone at 10 μg/kg body weight at 14 h before stripping. Fish were anesthetized (with 30 mg/l clove oil) and placed laterally on a table. A finger was inserted into the gonopore to stretch the opening slightly. A scalpel (with a straight blade narrower than the gonopore) was inserted carefully into the gonopore opening, and a 1.5–3 cm incision was made through the ventral area of the oviductal (Mullerian duct) wall. The scalpel was withdrawn and the incision probed with one finger to ensure that the opening was not obstructed. The fish was inverted and by slight pressure applied to the abdominal region by two individuals, the then flowed through the incised opening in the oviduct and out of the gonopore (Aramli et al., 2014).

2.4. Determination of semen density and osmolality

Sperm density was estimated using a Burker cell hemocytometer (Meopta, Czech Republic) at 200 × magnification on an Olympus BX50 phase contrast microscope (Olympus). Freezing point depression (as an indication of osmolality) was measured in duplicates for each sample with an osmometer (Osmomat 030-m, Berlin, Germany).

2.5. Effect of cryopreservation with different disaccharides extenders on semen motility and fertilization ability

Semen samples were diluted 1:5 in 9% methanol containing (i) 0.2 M maltose, (ii) 0.2 M trehalose, (iii) 0.2 M lactose and (iv) 0.2 M lactulose, and loaded into 0.25 ml plastic straws, equilibrated for 10 min on ice. Cryopreservation was performed using a freezing unit for straws which included an adjustable floating rack and Styrofoam box with an isolating Neopor block (MINITUB GmbH, Tiefenbach, Germany). Semen suspensions were placed in 0.5 ml straws (CRYO-VET, France) and suspended 3 cm above liquid nitrogen in a Styrofoam box for 10 min and then plunged into the liquid nitrogen. The samples were then thawed by immersion in a water bath at 40 °C for 4 s and semen was used to assess motility and fertilization ability. Additional samples were stored in liquid nitrogen for 5 weeks and then used for experiments (Section 2.6 below). The cryopreservation procedure described in this section also refers to the time zero of storage for the experiment described in Section 2.6.

2.6. Effect of post-thawing storage time on the motility of semen cryopreserved with different disaccharides extenders

Semen samples were cryopreserved as described above. Semen motility assessment was performed immediately after thawing (within 1–2 min) and after post-time storage. Therefore, data for time zero were obtained independently for previous experiment (Section 2.5) and analyzed statistically together with other time points for post-thawing storage. After thawing (40 °C, 5 s), semen was stored for 0, 15, 30 and 60, min at 4 °C in the freezing extender.

2.7. Semen motility analysis

Semen was diluted at a ratio of 1:50 with water from tanks in which the fish were kept, and sperm motility was immediately measured until cessation, using a CCD video camera (Sony, SSCDC50AP) mounted on a dark-field microscope (400 ×, Olympus CK2, Tokyo, Japan) and illuminated with a stroboscopic lamp (Chadwick- Helmut, 9630, USA) set to a flash frequency of 50 Hz. Video recordings were obtained (Sony SVHS, SVO-9500MDP) at 25 frames s^{-1} and analyzed to estimate sperm curvilinear velocity (VCL, μm s^{-1}) and the proportion (%) of motile sperm using Olympus micro-image software (Olympus Micro Image 4.0.1. for Windows, Hamburg Germany). To compute VCL and the proportion (%) of motile sperm, head tracks were generated from four successive video frames, and VCL was calculated as the length of the sperm head track divided by the time elapsed between the first and fourth video frame. In total, 10 to 25 sperm were evaluated for each frame. Sperm with a velocity lower than 3 μm s^{-1} were considered immotile and were excluded from further analysis (Rodina et al., 2008).